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Effect of cryoprotectants, equilibration periods and freezing rates on cryopreservation of spermatozoa of mahseer, *Tor khudree* (Sykes) and *T. putitora* (Hamilton)

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Abstract

A study was conducted to standardize a protocol for cryopreservation of spermatozoa of the endangered mahseer, *Tor khudree* (Sykes) and *T. putitora* (Hamilton). The suitability of the cryoprotectants, dimethyl sulphoxide (DMSO) and glycerol, and the combination of the two were tested. Four equilibration periods and four freezing rates were also tested for their standardization. A combination of 9% DMSO and 11% glycerol gave significantly higher mean percentage of hatching in both *T. khudree* (45.59 ± 1.86%) (control 71.08 ± 0.59%) and *T. putitora* (45.00 ± 1.25%) (control 73.48 ± 1.19%) among the eight different treatments. Among the four different equilibration periods tested, the equilibration period of 30 min^{-1} yielded the highest mean hatching percentage in *T. khudree* (39.46 ± 1.94%) (control 71.70 ± 0.61%) and *T. putitora* (38.28 ± 1.06%) (control 73.11 ± 0.82%). Freezing straws at a height of 8 cm above LN_2 surface for 10 min^{-1} gave higher hatching percentages for both *T. khudree* (41.75 ± 1.72%) (control 73.99 ± 1.17%) and *T. putitora* (41.34 ± 2.04%) (control 72.48 ± 1.51%). The study reports the superior performance of the combination of DMSO and glycerol for the first time.

Keywords: cryopreservation, mahseer, spermatozoa, cryoprotectants, equilibration time, freezing rate

Introduction

Mahseers, a group of important food and game fishes of India, formed a major fishery of the rivers and streams of the Shivalik Himalayas and the lower reaches of uplands of the deccan plateau (Mahanta, Kapoor, Pandey, Srivastava, Dayal, Patiyal, Joshi, Singh & Paul 1998). They are currently enlisted as a group of threatened fishes (Ogale 1994; Basavaraja & Keshavanath 2000). Cryopreservation of fish spermatozoa has many advantages over other forms of live gene banking and has gained much acceptance in the past few years (Lakra 1993; Rana 1995). However, detailed studies with respect to various parameters involved in cryopreservation of mahseer spermatozoa are lacking (Ponniah, Lakra & Ogale 1999a; Ponniah, Sahoo, Dayal & Barat 1999b; Basavaraja, Hegde, Akash & Udupa 2002). Even though few studies have been conducted on these lines (Basavaraja *et al.* 2002), no effort has been made so far to evaluate the effect of cryoprotectants in different combinations. Hence, two species of mahseer, viz., deccan mahseer, *Tor khudree* (Sykes), and golden mahseer, *T. putitora* (Hamilton), were selected for the standardization of cryopreservation protocol for the spermatozoa with respect to the effect of cryoprotectants, different equilibration times and freezing rates.

Materials and methods

The experiments were conducted at the mahseer hatchery complex of Tata Power Company, Lonavala, Maharashtra. Mature males and females of *T. khudree* were caught by overnight gill netting during the south-west monsoon season (July–August 2002). The brooders of golden mahseer, *T. putitora* were caught either by cast nets or by dragging with a net from the ponds at the farm during October–Decem-

ber 2002. The genital aperture of the fish was rinsed with sterile extender and wiped dry with a clean and slightly damp towel. The gametes were collected by gentle abdominal massage. The milt was collected in labelled, clean, dry and sterile graduated centrifuge tubes of 15 mL capacity. Milt samples with faecal matter or blood or other contaminants like scales were discarded. Milt was collected separately in different vials from individual males and stored on ice in a styrofoam box till further use, while the eggs were collected in clean, dry beakers of 250 mL capacity and were covered with aluminium foil and brought to the hatchery complex. The morphological evaluation of the milt involved recording the volume and pH (Leung 1991). The density of the spermatozoa was estimated using improved Neubauer Haemocytometer (Boeco, Hamburg, Germany) chamber following the method of Ax, Dally, Didion, Lenz, Love, Varner, Hafez and Bellin (2000). Similarly, the spermatocrit values were determined according to the method of Rakitin, Ferguson and Trippel (1999). A method involving two-step dilution of Billard, Cosson, Crim and Suquet (1995) was used for the estimation of motility for the fresh milt. The milt samples with $\geq$ 70% forwardly motile spermatozoa (evaluated using subjective estimation) were used for cryopreservation as done by Lahnsteiner, Weismann and Patzner (1997) and the rest of the samples were discarded.

Optimization of sperm to egg ratio

Standardization for the optimal sperm to egg ratio was carried out according to a modified method of Suquet, Billard, Cosson, Normant and Fauvel (1995). Ten-fold dilutions were made and triplicates were maintained for each dilution including the neat. Approximately 100 eggs were distributed into each of the Petri plates. The neat and the diluted milt were dispensed into labelled Petri plates with the eggs.

General protocol used during fertilization assays

In all the fertilization assays conducted, approximately 100 eggs were used per Petri plate. The fertilization involved no solution except the filtered pond water. One to two milliters of the pond water was added as an activating solution and mixing was done using a blunt glass rod with a flattened and smooth edge. After about 30–60 s, more filtered pond water was added to all the Petri plates to remove excess milt and for water hardening of the eggs. The total number of eggs in each Petri plate was counted and the Petri plates were placed into wooden trays with a nylon mesh at the bottom. The trays were floated over water in small cement cisterns of 2.5 m $\times$ 1.2 m $\times$ 0.75 m dimension. Fresh water was sprayed from both sides of the cement cistern over the hatching trays so as to provide aeration as well as to maintain the flow-through system of water supply. The fertilization percentage was estimated based on the number of eggs that had reached the blastula stage after 5–6 h of incubation. The eggs were incubated for 72 $\pm$ 6 h and the hatchlings were counted and hatching percentage was calculated.

Evaluation of the suitability of cryoprotectants and their concentration

Five cryoprotectants, namely, methanol, dimethyl sulphoxide (DMSO), glycerol, ethylene glycol, and polyethylene glycol were screened initially for their suitability at three different concentrations of 5%, 10% and 15% (v/v) according to the method of Fabbrocini, Lavadera, Rispoli, Sansone (2000). An aliquot of the fresh milt was also kept under refrigeration temperature for the same period, which served as control group. After 30 min^{-1} of storage at refrigeration temperature, about 1–2 μL of the milt from various treatment groups as well as the control group was taken with a micropipette tip (equated with a micropipette) and placed on a clean, non-greasy and dry glass slide which was pre-focussed on the stage of the microscope. About 8–10 μL of dechlorinated tap water or filtered pond water was placed next to it and both were mixed on the stage of the microscope and observed immediately, under $\times$ 40 after placing a cover slip. The motility scores were recorded as per the standard 0–5 point scale of Sanchez-Rodriguez and Billard (1977) (Table 2) and the two cryoprotectants with higher percent motility were selected. Those concentrations of the cryoprotectants that yielded less motility(15%) or no motility ($<$7%) were rejected and were tested for their efficacy at other suitable concentrations ranging from 7% to 14% (v/v) successively differing by one unit. Only two (8% and 9% DMSO; 10% and 11% glycerol) of the eight best performing concentrations were used per cryoprotectant for the subsequent cryopreservation experiments. Here again, the motility percentages were estimated using the above method.

Experimental plan

To utilize the small sample size that was available for both *T. khudree* and *T. putitora*, the successive elimination procedure was followed to determine a suitable combination of cryoprotectant, equilibration period and freezing rate of cryopreservation protocol for these endangered species. The milt was cryopreserved with three main variables, namely, cryoprotectants (eight treatments), equilibration time (four treatments) and freezing rate (four treatments).

The evaluation of the cryopreservation success based on the percentage of hatching

In the first phase, the best cryoprotectant was identified from among the eight individual/combination of the cryoprotectants, viz., 8% DMSO, 9% DMSO, 10% glycerol, 11% glycerol, 8% DMSO+10% glycerol, 8% DMSO+11% glycerol, 9% DMSO+10% glycerol and 9% DMSO+11% glycerol, keeping the equilibration period fixed at 30 min^{-1} and a freezing rate of 5 cm and 10 min^{-1} over LN_2 surface. In the second phase, equilibration was optimized for the best cryoprotectant, keeping the freezing rate constant. Four equilibration periods, viz., 0, 30, 60 and 120 min^{-1}, were tested. In the third phase, the best freezing rate was identified for the best cryoprotectant and the optimal equilibration period. Four different freezing rates, viz., heights of 1, 5, 8 and 10 cm over LN_2 surface for 10 min^{-1}, were tested.

Cryopreservation and thawing

Milt samples from nine males were pooled in equal ratios for each species of mahseer after the initial milt quality analysis. All the materials used during cryopreservation trials, viz., straws, sealing powder, beaker, extender solution and the cryoprotectants, were chilled before use and care was taken to maintain the same temperature as that of milt. Based on the concentration of spermatozoa in the pooled milt as well as on the results of the trials on optimization of sperm to egg ratio, the milt was diluted with the diluent (modified BWW extender and the cryoprotectant) (Table 1) (Biggers, Whitten & Whittingham 1971) in the ratio of 1:100 (v/v). The diluted milt was filled into French medium (0.5 mL) straws and the straws were sealed with PVA powder. The straws were left in the chilled water till the desired equilibration was achieved, which also removed the excess sealing powder.

Table 1 Chemical composition of the modified BWW (Biggers *et al.* 1971) extenders

Constituent chemical compound	mM
NaCl	95
KCl	48
$CaCl_2 \cdot 2H_2O$	1.7
$NaHCO_3$	25
$MgSO_4 \cdot 7H_2O$	–
$MgCl_2$	–
NaH_2PO_4	–
Fructose	27.78
Mannitol	27.47
Sodium citrate (tribasic)	17.01
pH	8.0

After the respective equilibration periods, the straws were frozen at the required heights above LN_2 surface with the help of the rack stand. The rack stand was kept in a styrofoam box with LN_2 for freezing. The straw racks and the rack stands were designed and fabricated in the laboratory and were made of aluminium. The straw rack had a dimension of 28 cm × 8.5 cm with a provision to hold about 42 straws. The rack stand had a dimension of 52 cm × 29 cm × 20.5 cm with 12 pairs of vertical bars with holes on either side at every 1 cm interval so as to hold the straw rack at the desired height over the LN_2 surface. The position of the straw rack was fixed using two aluminium rods of 2 mm diameter and 30 cm length. After freezing, the straws were removed with forceps, put into goblets and immersed into cryocans. The studies were conducted three times for both *T. khudree* and *T. putitora* with triplicates in each experiment along with the control. The milt was thawed using a thermostat regulated water bath at 37–38 °C for 10 s and the contents after thawing were poured onto the eggs in the Petri plate. One straw was used per Petri plate which contained nearly 100 eggs. The rest of the procedure was conducted as explained in the general protocol.

Statistical analysis

The calculated means were expressed as mean standard error. Normality of the data was tested and wherever needed arcsine and logarithmic (to the base 10) transformations were carried out. Analysis of variance was performed to find out the significant

differences between the treatments at a significance level of $P < 0.05$ (Sokal & Rohlf 1998). All the statistical analyses were performed using SAS Package (Version 8.2).

Results

Assessment of milt quality

The average volume of milt in case of *T. khudree* and *T. putitora* was 2.31 ± 0.52 and 2.10 ± 0.40 mL respectively. The pH of milt ranged from 7.8 to 8.2 in both the species. The concentration of spermatozoa and the spermatocrit values were $3.88 \pm 0.14 \times 10^7$ spermatozoa mL^{-1} and 66.89 ± 1.40% for *T. khudree* and $3.92 \pm 0.16 \times 10^7$ spermatozoa mL^{-1} and 69.22 ± 1.88% for *T. putitora* respectively. The mean motility percentage of spermatozoa in fresh milt of *T. khudree* was 95.01 ± 0.85% while it was 95.18 ± 0.29% for *T. putitora*.

Selection of suitable cryoprotectants and optimization of their concentration

In both the species, highest motility was observed in glycerol at 10% level with a motility score of 3 for both the species followed by DMSO, which gave a score of 2 for both the species at 10% level (Table 2). There was either gel formation or poor or no motility when using other concentrations of DMSO or glycerol or other types of cryoprotectants. In more detailed investigations it was found that DMSO gave the highest motility score of 3 at concentrations of 8% and 9% followed by a motility score of 2 at concentrations of 10%, 11% and 12% levels. Glycerol gave the highest motility score of 3 at concentrations of 10% and 11% (Table 3). The other tested concentrations were not found suitable in both species.

Cryopreservation

The optimal sperm to egg ratio was $3.88 \pm 0.14 \times 10^3$ and $3.92 \pm 0.16 \times 10^3$ spermatozoa egg^{-1} in *T. khudree* and *T. putitora* respectively.

Effect of cryoprotectants

In both species the highest mean percentage of fertilization was recorded at DMSO concentrations of 8% (73.5 ± 1.1% for *T. khudree* and 73.7 ± 1.7% for

Table 3 Motility scores of spermatozoa of *Tor* species treated with DMSO and glycerol at different concentrations*

Cryoprotectant concentration (v/v) (%)	DMSO		Glycerol		Control†	
	T. khudree	*T. putitora*	*T. khudree*	*T. putitora*	*T. khudree*	*T. putitora*
7	MBA	MBA	MBA	MBA	4	4
8	3	3	MBA	MBA	–	–
9	3	3	2	2	–	–
10	2	2	3	3	–	–
11	2	2	3	3	–	–
12	2	1	2	2	–	–
13	1	1	2	1	–	–
14	1	1	1	1	–	–

*30 min^{-1} with modified BWW at refrigeration temperature.
†In control, fresh milt was diluted with only BWW extender.
MBA, motility before activation; DMSO, dimethyl sulphoxide.

Table 2 Motility scores of spermatozoa of *Tor* species treated with varying concentrations of cryoprotectants*

Species	DMSO (%)			Methanol (%)			Glycerol (%)			EG (%)			PEG (%)			Control†
	5	10	15	5	10	15	5	10	15	5	10	15	5	10	15	
T. khudree	MBA	2	1‡	MBA	1‡	1‡	MBA	3	0	1‡	1	1	0‡	0‡	0‡	4
T. putitora	MBA	2	1‡	MBA	0‡	0‡	MBA	3	0	0‡	0‡	0‡	0‡	0‡	0‡	4

5	Quick wave motions: >80%
4	Trace swirls: 60–80%
3	Individual motility seen: 40–60%
2	Motility: 20–40%
1	Motility: <20%
0	Zero motility

*30 min^{-1} with modified BWW at refrigeration temperature.
†In control, fresh milt was diluted with modified BWW only.
‡Gel formation.
MBA, motility before activation; DMSO, dimethyl sulphoxide.

Table 4 Effect of different cryoprotectants on fertilization and hatching percentages in *Tor khudree* and *T. putitora*

	Percent fertilization (mean ± SE) (absolute values)		Percent hatching (mean ± SE) (absolute values)	
Cryoprotectant	***T. khudree***	***T. putitora***	***T. khudree***	***T. putitora***
DMSO 8% (D)	73.5 ± 1.1^{a}	73.7 ± 1.7^{a}	32.3 ± 0.5^{b}	32.4 ± 0.5^{b}
DMSO 9% (D)	64.2 ± 3.5^{bc}	64.8 ± 1.4^{b}	30.6 ± 1.4^{b}	33.1 ± 1.6^{b}
Glycerol 10% (G)	48.7 ± 1.8^{e}	51.6 ± 2.2^{c}	25.9 ± 1.6^{c}	26.0 ± 1.5^{c}
Glycerol 11% (G)	61.5 ± 0.7^{c}	63.7 ± 0.7^{b}	23.4 ± 1.1^{c}	22.9 ± 0.9^{c}
8% D+10% G	53.1 ± 0.9^{d}	54.5 ± 1.7^{c}	28.9 ± 1.3^{b}	29.5 ± 1.8^{bc}
8% D+11% G	49.0 ± 0.8^{e}	53.2 ± 1.7^{c}	30.1 ± 1.1^{b}	30.7 ± 1.5^{b}
9% D+10% G	68.1 ± 1.3^{b}	66.8 ± 3.5^{b}	42.2 ± 1.2^{a}	42.1 ± 1.6^{a}
9% D+11% G	73.0 ± 2.0^{a}	65.5 ± 2.9^{b}	45.6 ± 1.9^{a}	45.0 ± 1.3^{a}
Control	86.1 ± 1.3	84.3 ± 1.9	71.1 ± 0.6	73.5 ± 1.2

Values with the same superscripts do not have significant difference within the respective fertility measures and within the species.

Table 5 Effect of different equilibration periods on fertilization and hatching percentages in *Tor khudree* and *T. putitora*

	Percent fertilization (mean ± SE) (absolute values)		Percent hatching (mean ± SE) (absolute values)	
Equilibration time	***T. khudree***	***T. putitora***	***T. khudree***	***T. putitora***
0 min^{-1}	42.4 ± 1.7^{c}	42.5 ± 1.2^{c}	18.5 ± 0.6^{c}	21.9 ± 1.6^{c}
30 min^{-1}	70.5 ± 1.3^{a}	73.2 ± 0.9^{a}	39.5 ± 1.9^{a}	38.3 ± 1.1^{a}
60 min^{-1}	61.7 ± 0.9^{b}	63.8 ± 1.3^{b}	27.1 ± 1.1^{b}	28.2 ± 1.9^{b}
120 min^{-1}	67.4 ± 1.1^{a}	65.6 ± 0.6^{b}	25.9 ± 1.2^{b}	23.5 ± 1.8^{c}
Control	86.0 ± 1.1	83.2 ± 1.5	71.7 ± 0.6	73.1 ± 0.8

Values with the same superscripts do not have significant difference within the respective fertility measures and within the species.

T. putitora). The mean percentage of fertilization in the control was 86.1 ± 1.3% for *T. khudree* and 84.3 ± 1.9% for *T. putitora* (Table 4).

The significantly highest mean percentage of hatching was recorded in a mixture of 9% D+11% G (45.6 ± 1.9% in *T. khudree* and 45.0 ± 1.3% in *T. putitora*) (Table 4). The lowest mean percentage of hatching was observed in 10% glycerol. The percentage of hatching in the control was 71.08 ± 0.59% for *T. khudree* and 73.5 ± 1.2% for *T. putitora* (Table 4).

Effect of equilibration period

The mean percentage of fertilization was highest at an equilibration period of 30 min^{-1} and lowest at 0 min^{-1} (Table 5). This was similar for both the species.

The mean percentage of hatching was also significantly higher at an equilibration period of 30 min^{-1} in both the species (Table 5). The lowest hatching percentage was observed at equilibration periods of 0 min^{-1} (Table 5).

Effect of freezing

In *T. khudree* and *T. putitora*, the highest mean percentage of fertilization and hatching was observed at a freezing level of 8 cm above the surface of LN_2 for 10 min^{-1} (Table 6). There was a significant difference between the different treatments ($P < 0.05$).

Discussion

In the present study, spermatozoa were activated in ≤ 5% glycerol and lost motility within 30 s. However, cryoprotectants should not activate the spermatozoa and should not be toxic and irreversibly inhibit sperm motility. Among the eight cryoprotectants that were tested for their efficacy, 9% DMSO+11% glycerol was found to be the most suitable cryoprotectant for *T. khudree* and for *T. putitora* in the present study. This is possibly because of their synergistic effect which might result in higher protection against cryo-injuries. Dimethyl sulphoxide is observed to be a better cryoprotectant mainly because it penetrates and

Table 6 Effect of different freezing rates on fertilization and hatching percentages in *Tor khudree* and *T. putitora*

	Percent fertilization (mean ± SE) (absolute values)		Percent hatching (mean ± SE) (absolute values)	
Freezing	***T. khudree***	***T. putitora***	***T. khudree***	***T. putitora***
1 cm 10 min^{-1}	29.7 ± 2.4^{c}	28.7 ± 1.8^{c}	21.0 ± 0.9^{c}	20.7 ± 1.3^{c}
5 cm 10 min^{-1}	70.9 ± 1.5^{a}	69.9 ± 2.4^{a}	36.9 ± 2.2^{ab}	35.8 ± 2.5^{ab}
8 cm 10 min^{-1}	72.7 ± 1.1^{a}	72.8 ± 1.9^{a}	41.8 ± 1.7^{a}	41.3 ± 2.0^{a}
10 cm 10 min^{-1}	52.7 ± 2.6^{b}	49.2 ± 1.6^{b}	32.0 ± 1.8^{b}	31.4 ± 1.4^{b}
Control	84.6 ± 1.0	81.3 ± 0.8	73.6 ± 1.2	72.5 ± 1.5

Values with the same superscripts do not have significant difference within the respective fertility measures and within the species.

leaves the cells much faster than glycerol. However, DMSO is found to be toxic when used at concentrations above 20% (v/v) or when the equilibrations time is extended (Stoss & Refestie 1983).

When glycerol was used at 10% and 11%, a hatching percentage ranging from 22.9 ± 0.9 to 26.0 ± 1.5 was obtained. In another study, Piironen (1993) used 20% glycerol in arctic charr with an average percentage of fertilization of 75% of control. Piironen and Hyvarinen (1983) and Piironen (1987) also used 20% glycerol and obtained high fertilization percentages. Conget, Fernandez, Herrera and Minguell (1996) used 13.82% glycerol and obtained >40% motility in spermatozoa in case of rainbow trout. However, Ritar (1999) used 18.4% glycerol in case of striped trumpeter with only 13% fertilization rate. In our study, DMSO at concentrations of 8% and 9% levels (v/v) differed significantly from each other with respect to the fertilization rates, while DMSO at a concentration of 15% was not suitable. This contradicts the observations of Basavaraja *et al.* (2002) who recorded a high hatching rate with 15% DMSO. Gupta and Rath (1993) reported a high hatching rate of 30–40% with 15% DMSO in Indian major carps. Ponniah *et al.* (1999a) reported that in case of *T. khudree*, 10% methanol resulted in best percentage of fertilization (23.8%) and 10% DMSO in best percentage of hatching (13.6%). However, rates of fertilization and hatching obtained by these workers were very much lower when compared with our results.

An equilibration period of 30 min^{-1} was optimal for the cryopreservation of spermatozoa in *T. putitora* and *T. khudree* as this treatment resulted in highest post-thaw fertility parameters. The hatching percentages were significantly lower at 0 min^{-1} equilibration as well as 120 min^{-1} equilibration. On the contrary, Basavaraja *et al.* (2002) found that the fertilization rates were not influenced by equilibration time in *T. khudree*. Our results support the findings of Ponniah *et al.* (1999a) who reported high hatching rates at equilibration periods of 45 min^{-1} and reduced hatching rates at equilibration time of 120 min^{-1} in *T. khudree*. However, contradictory to our results, Ponniah *et al.* (1999b) obtained highest hatching percentage with an equilibration period of 60 min^{-1} in *T. putitora*. Ashwood-Smith (1986) reported that, at equimolar concentrations, cryoprotection by glycerol is almost identical to DMSO, but because of its slower permeation into cells, glycerol takes longer time to reach equilibrium than DMSO. In the present study, it was also found that 0 min^{-1} equilibration time resulted in very low hatching percentage. Hence, too short and too long durations of equilibration time were not found suitable for cryopreservation. Chao, Chen and Liao (1975) and Billard (1978) suggested that the equilibration time should be kept to a minimum mainly to minimize the cryoprotectant toxicity.

Concerning the freezing conditions, the results of the present study are in agreement with those of Basavaraja *et al.* (2002), who also obtained high percentage of fertilization (98.15–99.56%) and high hatching rates (40.81%) in *T. khudree* by freezing the straws at a height of 5 cm over LN_2 for 10 min^{-1}. Surprisingly, in a paradoxical finding, Alderson and Macneil (1984) recorded that freezing rates had no effect on post-thaw fertility over a range of freezing rate of 20–140 °C min^{-1} in case of Atlantic salmon, *Salmo salar*. In the present study, decreased fertilization percentage and hatching percentage were observed at freezing levels of 1 cm as well as 10 cm above the surface of LN_2, indicating that too high and too low freezing rates are detrimental for cryopreservation of spermatozoa of the two mahseer species. Therefore, the optimum freezing rate is at a level of 5–8 cm above the surface of LN_2 for 10 min^{-1}.

The statistical analysis of the fertility trials revealed that there was no significant difference between the two species of mahseer, viz., *T. khudree* and *T. putitora*, with respect to the different treatments.

Hence, we are of the opinion that an optimized cryopreservation protocol for a particular fish species belonging to a genus can be used for another species of the same genus. The different results obtained for *T. khudree* and *T. putitora* by other workers (Ponniah *et al.* 1999a, b; Basavaraja *et al.* 2002) might be because of the fact that the quality of the spermatozoa is highly variable and depends on various external factors like the feeding regime, the quality of food and the rearing temperature of the males (Billard, *et al.* 1995; Labbe & Maisse 1996; McNiven, Pustowka, Richardson & Lall 1999). Amann (1991) observed that the post-thaw performance of the milt varies between individual males. Hatching percentages were lower than fertilization percentages in the present study. We suggest that the spermatozoa with cryodamages initiate development of the embryo; however, because of altered paternal chromosomes, the embryonic development stops in later stages of development (Labbe, Martoriati, Devaux & Maisse 2001; Gwo, Wu, Chang & Cheng 2003). As the post-thaw performance of cryopreserved spermatozoa of fish varies among individuals of a species (Amann 1991; Babiak, Glogowski, Luczynsky, Goryczko, Dobosz & Kuzminski 1998) the males with suitable sperm quality can be identified by chill-injury test (Tomar 1997) and can be tagged. There are reports confirming the significance of dietary lipids in influencing the post-thaw fertility parameters of the cryopreserved spermatozoa (McNiven *et al.* 1999), and hence the selected males in the broodstock ponds should be fed with a specially formulated feed so as to get high post-thaw fertility rates. This practice could be very effective in case of commercially important species of fish.

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